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Original Paper

Effect of 1,25-$(OH)_2D_3$on Proliferation of Fibroblast-Like Synoviocytes and Expressions of Pro-Inflammatory Cytokines through Regulating MicroRNA-22 in a Rat Model of Rheumatoid Arthritis

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Key Words
Rheumatoid arthritis • 1,25-dihydroxyvitamin D_3 • MicroRNA-22 • Fibroblast-like synoviocytes • Pro-inflammatory cytokine • Collagen induced arthritis

Abstract
Objective: This study aims to investigate the regulatory mechanism of 1,25-$(OH)_2D_3$ on the proliferation of fibroblast-like synoviocytes (FLS) and expressions of pro-inflammatory cytokines in rheumatoid arthritis (RA) rats via microRNA-22 (miR-22). ***Methods:*** A rat model of RA was established with a subcutaneous injection of type II collagen. After treated with different concentrations of 1,25-$(OH)_2D_3$ the proliferation of FLS was estimated by the MTT method, and the optimal concentration of 1,25-$(OH)_2D_3$ was selected for further experiments. Cell proliferation was detected by MTT. Cell cycle and apoptosis wereanalyzed by FCM.The IL-1ß, IL-6, IL-8, and PGE2 protein expressions were determined by ELISA, and MMP-3, INOS, and Cox-2 mRNA expressions were measured by qRT-PCR. ***Results:*** The rat model of RA was successfully established. Compared with the blank group, the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups exhibited higher proliferation inhibition and apoptosis rates, lower levels of pro-inflammatory cytokines (IL-1ß, IL-6, IL-8, and PGE2), and decreased mRNAexpressions of MMP-3, INOS, and Cox-2. The miR-22 mimics group had lower proliferation inhibition and apoptosis rates, elevated expressions of pro-inflammatory cytokines and MMP-3, INOS, and Cox-2 than the blank group. In contrast to the 1,25-$(OH)_2D_3$ group, the proliferation inhibition and apoptosis rates were down-regulated, and the expressions of pro-inflammatory cytokines and MMP-3, INOS, and Cox-2 were up-regulated in the 1,25-$(OH)_2D_3$ + miR-22 mimics group. ***Conclusion:*** Our study demonstrated that 1,25-$(OH)_2D_3$ inhibits the proliferation of FLS and alleviates inflammatory response in RA rats by down-regulating miR-22.

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Introduction

Rheumatoid arthritis (RA), affecting 0.5-1.0% of the adult population worldwide, is a complicated autoimmune disease with systemic sequelae and is characterized by a chronic inflammation of synovial joints and bone erosion [1]. Environmental and genetic factors are two major aspects of RA and account for approximately 60% and 40% of the risk for developing RA, respectively [2]. Although the mechanism underlying RA remains unknown, fibroblast-like synoviocytes (FLS) have been reported to play a critical role in the pathogenesis of RA [3, 4]. In the normal synovium, FLS are a highly differentiated unicellular cell type, governing the provisions of support, nourishment, and lubrication of the joint tissue [5]. However, when an inflammatory response occurs, FLS become hyperplastic, invasive, and highly migratory, contributing to cartilage destruction and thus, RA [6, 7]. Van Hamburg et al. reported that the combination of neutralizing TNF activity and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) contribute to controlling human Th17 activity, which induces a pro-inflammatory feedback loop on RA synovial fibroblast interactions and inhibits synovial inflammation [8]. However, the specific mechanism of this function requires a further investigation.

To the best of our knowledge, microRNAs (miRNAs) are a class of small, non-coding, single-standed RNAs of ~22 nucleotides and are involved in a series of biological processes, such as cell proliferation, differentiation, and apoptosis [9]. MiRNA-22 (miR-22) is located on chromosome 17p13.3 (close to *TP53*) between markers D17S1866 and D17S1574, and resides in the exon 2 region of the non-coding C17 or f91 gene. MiR-22 is regarded as a suppressor of tumorigenesis [10]. Lin *et al*.found that the dysfunction of miR-22 expression can facilitate the inflammation process of RA through affecting the p53 mutation in synovial cells [11]. Also, miR-22 shows an anti-apoptotic effect in myocardial ischemia–reperfusion injuries and Huntington's disease [12, 13]. Interestingly, miR-22 is induced by vitamin D [14], and the 1,25-$(OH)_2D_3$ is the most active vitamin D metabolite that inhibits osteoblast mineralization and represses the osteocalcin gene [15]. Furthermore, Gopinath *et al*. reported that 1,25-$(OH)_2D_3$ is an anti-osteoporotic agent in RA due to its immunomodulatory functions [16]. Therefore, this study intended to identify the influence of 1,25-$(OH)_2D_3$ on the cell proliferation of FLS and cytokines in RA and shed light on how miR-22 affects this process.

Materials and Methods

Subjects

This animal experiment was approved by the Ethics Committee of the Center of Nephrology, the First Affiliated Hospital Xi'an Jiaotong University, and all of the experimental procedures conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Sixty healthy male Sprague Dawley (SD) rats of clean grade (4-6 weeks, weighing 150 - 190 g) were included, and these rats were purchased from the Research Animal Care Committee of Nanjing Medical University. All rats were fed in a specific pathogen free (SPF) animal room with a humidity of 50% at 24°C and a day/night period of 12/12 h.

A rat model of rheumatoid arthritis (RA)

At first, 60 rats were randomly assigned into the control group (n = 20) and the model group (n = 40). After one week of living in the SPF animal room, the rats in the model group were intradermally injected with 0.1 mL emulsion of bovine type II collagen (Sigma Co., Ltd., St Louis, MO, USA) on their left hind foot. The emulsion was prepared as following steps: the bovine type II collagen was dissolved in 0.01 mol/L acetic acid at a final concentration of 2 mg/ml and were stored overnight; and then complete freund's adjuvant(Sigma Co., Ltd, St Louis, MO, USA) and the dissolved bovine type II collagenwere well mixed at a ratio of 1:1 in a high-speed homogenate machine. After beingdisinfected with 75% alcohol wipes and the emulsion of bovine type II collagen was injected into rats using a micro-syringe.One week later, 0.1 mL of emulsion was injected into the tail of each rat to intensify the immunization. Rats in the control group were injected with Freund's adjuvant into their feet and tails using the same method as the model group.

Observation and RA model verification

During the experimental process, the color of hind feet skin and ankle joint skin, skin temperature, hind feet action, and ankle joint swellingwere recorded. On the 7th day of model construction, the RA scores of two hind feet of rats were measured according to the identification of RA in rats [17]. The meanRA score ofhind feet of each rat was obtained, and then the mean RA score of each group was calculated. The RA score was measured as follows: 0 score, no arthritis symptom was observed in hind feet; 1 score, one to two joints in hind feet turned red with soft tissue swelling; 2 scores, three to four joints in hind feet turned red with soft tissue swelling; 3 scores, five or even more joints in hind feet turned red with soft tissue swelling; 4 scores, severe arthritis symptoms were observed in hind feet. On the 28th day of the experiment, 5 rats of the control group and 5 rats in the model group were killed, and then their joints were separated immediately and fixed informaldehyde, followed by decalcification and hematoxylin and eosin (HE) staining. If infiltration of inflammatory cells was clearly observed in the pathological section of joints, the RA model was regarded as successfully established.

Isolation and identification of fibroblast-like synoviocytes (FLS)

After successful construction of RA model, the synovial tissues of joints were obtained under sterile conditions, after which the adipose tissue and fiber texture were eliminated and then were digested at 37°C for 30 min with 1 mg/ml of type I collagen enzyme (Gibco, BRL, Grand Island, New York, USA). The synovial tissues were filtered using a 200 mesh strainer. The collagen induced arthritis (CIA) FLS were obtained by centrifugation at a rate of 1000 rpm for 5 min. Then FLS were cultured in an incubator and were sub-cultured at a ratio of 1:2 once cells had grown all over the plate.And cells of 3 to 7 generations were used for further experiments.

The cell suspension was added into a 24-well plate (with slide) at a density of 2×10^4 / mL and cultured in an incubator for 48 h. With the supernatant removed, the plate was fixed in formalin for 30 min and rinsed with phosphate buffered saline (PBS). With the addition of the rabbit anti-mouse vimentin monoclonal antibody (Abcam, Co., Ltd., Cambridge, MA, USA) or the mouse anti-human CD68 monoclonal antibody (Abcam, Co., Ltd., Cambridge, MA, USA), the plate was incubated at 37°C for 1 h. After that, it was rinsed with PBS, and was added with the second antibody (Santa cruz biotechnology, Inc., santacruz, CA, USA). It was thenincubated at 37°C for 20 min and rinsed with PBS again. Then, diaminobenzidine (DAB) was applied for coloration, followed by sealing, observation and photographing. PBS was used to replace the primary antibody (vimentin antibody) as the negative control.

Methyl thiazolyl tetrazolium (MTT)assay

FLS in the logarithmic growth phase were collected for preparation of a cell suspension. They were seeded into a 96-well plate (1 × 105 cells / ml, 100 μl / well) and cultured for 24 h. After that, different concentrations of 1,25-$(OH)_2D_3$ (10^{-9}, 10^{-8}, and 10^{-7} mol / L) were added into the plate and the cells were continuously cultured in an incubator (5% CO_2) at 37°C for 24 h or 48 h. After the addition of 20 μl of MTT solution (5 mg / ml) into each well, cell were cultured for 4 h. The supernatant was then removed and 150 μl of dimethylsulfoxide (DMSO) was added into each well and shaken for 15 min to fully dissolve the crystals. The enzyme-linked immune detector (Themo Fisher Scientific, Waltham, MA, USA) was used to detect the optical value (A570)at 579 nm of each well, based on which the inhibitory rate of cell growth was calculated. The above step was performed three times to obtain the mean value. The formula: inhibitory rate of cell growth (%) = (1 – A570 of the model group / A570 of control group) × 100%. The concentration of 1, 25-$(OH)_2D_3$with an optimal inhibitory effect on FLS proliferation was selected for further experiments. The MTT assay was used to detect the FLS proliferation in this experiment. The procedures were the same as above for detecting the inhibitory rate of cell growth.

Cell transfection and grouping

According to the different treatments, FLS were divided into 7 groups in the study: the blank group, the 1,25-$(OH)_2D_3$ group, the miR-22 mimics group, the miR-22 mimics negative control (NC) group, the miR-22 inhibitors group, the miR-22 inhibitors NC group, and the 1,25-$(OH)_2D_3$ + miR-22 mimics group. In the 1,25-$(OH)_2D_3$ group, FLS were treated with the optimal concentration of 1,25-$(OH)_2D_3$ culture solution.In the miR-22 mimics, miR-22 mimics NC, miR-22 inhibitors,and miR-22 inhibitors NC groups, miR-22 mimics, miR-22 mimics NC, miR-22 inhibitors and miR-22 inhibitors NC (Shanghai GenePharmaBiopharmacy Co.,

Ltd. Shanghai, China) were respectively transfected into FLS. After being transfected with miR-22 mimics for 12 h, the optimal concentration of 1,25-$(OH)_2D_3$ culture solution was added into FLS in the 1,25-$(OH)_2D_3$ + miR-22 mimics group. In the blank group, cells were cultured withmedium only. Cell transfection was conducted using the liposome 2000 kit (Invitrogen Inc., Carlsbad, California, USA).

Flow cytometry (FCM)

FCM was applied to detect the distribution of cell cycle for each group using a cell cycle detection kit (R&D Systems Inc., Minneapolis, Minnesota, USA). The mean value was recorded after three repeated trials. FLS were collected,and rinsed with ice-cold 1 × PBS three times. The supernatant was discarded after centrifugation. Then, the cells were re-suspended with 0.5 ml 1 × PBS, addedwith 3.5 ml of 70% pre-cooled ethyl alcohol, and stored at 4°C overnight. Cells were fixed with ethyl alcohol and centrifuged, after which the supernatant was removedand cells were rinsed with 1 × PBS three times. After being re-suspended with 1 ml PI/Triton X-100 staining fluid (20 μg PI/0.1% Triton X-100) containing 0.2 mg of RNase A, the cells were stained at 37°C for 15 min. The cell cycle was detected on a flow cytometer.

The terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) kit (R&D Systems Inc., Minneapolis, Minnesota, USA) was employed to measure the apoptosis rate of each group. FLS were seeded into a 6-well plate and stained according to the kit instructions. The cell apoptosis was observed under a light microscope, and cells with brown particles in their nucleus were regarded as positive cells. Five fields were selected for cells in each glass slide with high power microscopes (400×). The numbers of apoptotic positive cell nuclei and total cell nuclei were separately counted. The apoptotic index (AI) was determined by the ratio of the number of apoptotic positive cell nuclei to the number of total cell nuclei. This experiment was repeated three times and the mean value was obtained.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted with the Trizol kit (Invitrogen Co., Carslbad, CA, USA) from FLS in the seven groups (the blank, 1,25-$(OH)_2D_3$, miR-22 mimics, miR-22 mimics NC, miR-22 inhibitors, miR-22 inhibitors NC, and 1,25-$(OH)_2D_3$ + miR-22 mimics groups). The spectrophotometer provided by the Bio-Rad biological technology Co., Ltd (Hercules, CA, USA) was used to measure the D260 / D280 ratio and the concentration of RNA. The D260 / D280 ratio at 1.8 to 2.0 was preserved for later experiments. The PCR primers were synthesized by Shanghai SangonBiological Engineering Technology & Services Co., Ltd. (Shanghai, China) Co., Ltd, andprimer sequencesare shown in the Table 1. The cDNA reversion transcription and qRT-PCR system were prepared according to the instructions of the cDNA reverse kit (Takara Bio Inc., Tokyo, Japan) and the qRT-PCR kit (Tiangen Biotechnology Co., Ltd., Beijing, China). Reaction condition: pre-denaturation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60-66°C for 20-32 s in total. The mRNA expressions of miR-22, matrix metalloproteinase 3 (MMP-3), inducible nitric oxide synthase (INOS), and cyclooxygenase-2 (Cox-2) mRNA were separately tested.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of the seven groups were collected and underwent centrifugation at 12000 rpm for 10 min. Then, the protein expressions of IL-1β, IL-6, IL-8, and prostaglandin E2 (PGE2) were detected. ELISA was performed in line with the ELISA kit instructions (Cusabio Biotech Co., Ltd., Wuhan, China). At least a three-well parallel testing was implemented for each specimen.

Table 1. The primer sequences for quantitative real-time polymerase chain reaction. Note: MMP3, matrix metalloproteinase 3; INOS, inducible nitric oxide synthase; Cox2: cyclooxygenase-2

Gene		Sequence
GAPDH	F	ACAGCAACAGGGTGGTGGAC
	R	TTTGAGGGTGCAGCGAACTT
microRNA-22	F	ACACTCCAGCTGGGTTCGACGGTCAACTTC
	R	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGTTCT
MMP3	F	CATAATACACAGCTGACCTGTATAA
	R	ATTTAAGAAATCATAGATAACAGTTACTTA
INOS	F	GAGGATCCCCGGGTACCGGTCGCCAGGATGGCTTG
	R	TCACCATGGTGGCGACCGGGAGTCTTGTGCCTTTG
Cox2	F	CAATCTGGCTGAGGGAACACAACA
	R	ATCTGCCTGCTCTGGTCAATGGA

Statistical analysis

SPSS 17.0 software (SPSS Inc. IBM, Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation (mean ± SD). One-way analysis of variance (ANOVA) was applied in statistical analysis and the Bonferroni method was used for pairwise comparison between two items. $P< 0.05$ indicates statistical significance.

Results

Symptoms in the hind feet and joint of rats and histological features of their joints in the RA model

In the early stage of RA model, the rats in the model group were less active and had less food and water intake with more symptoms of limb edema and hair tarnishing than the rats without the injection of type II collagen in the control group. Also, some rats in the model group showed acute inflammation signs that joint skin turned red, light and congestion, and joint motion was blocked withdifficulty in bearing loads. No obvious abnormity was found in the rats of the control group. The RA score in the control group was 0 score, whereas the RA score of the model group was gradually increased. On the 28th day, red joints and severe soft tissue swelling were observed in the model group, and the RA score reached its highest level (Fig. 1). According to the results of HE staining, the cartilage of rats turned a faint pink with sclerotin in deep color, clearly visiblejoint gap and no inflammatory cell in the joint gap in the control group. However, there was a mass of inflammatory cells in sheet forms and dark blue color in rats of the model group. Due to arthritis, the joint structure became to be unclear, inflammatory cells eroded into sclerotin, pink cartilage and dark red sclerotin were hardly seen, and the joint gap was almost disappeared which was filled by inflammatory cells.

Immunohistochemicalexpressions of vimentin and CD68 in FLS

Specially expressed and non-expressed molecules in FLS were detected by immunohistochemistry staining and observed under a microscope. Cells with theaddition of vimentin antibody showed saffron yellowgranules, which suggested that vimentin is positively expressed in these cells. Cells with the addition of CD68 antibody showed purplegranules, which suggested thatno CD68 expression was observed in these cells. The cells were shuttle-shaped and arranged irregularly and conformed to the molecular characterization and features of FLS. In the negative control cases, cells exhibited a negative result (Fig. 2).

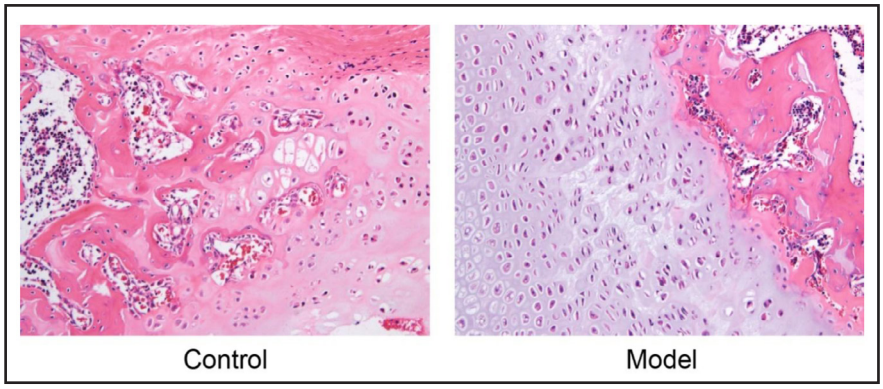

Fig. 1. Identification of collagen induced arthritis rat model by HE staining.

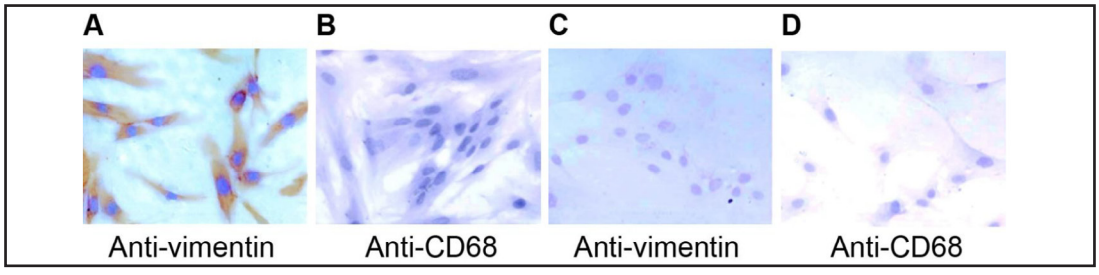

Fig. 2. Identification of fibroblast-like synoviocytes. (A) Anti-vimentin staning of fibroblast-like synoviocytes; (B) Anti-CD68 staning of fibroblast-like synoviocytes; (C) Anti-vimentin staning of negative control cells; (D) Anti-CD68 staning of negative control cells.

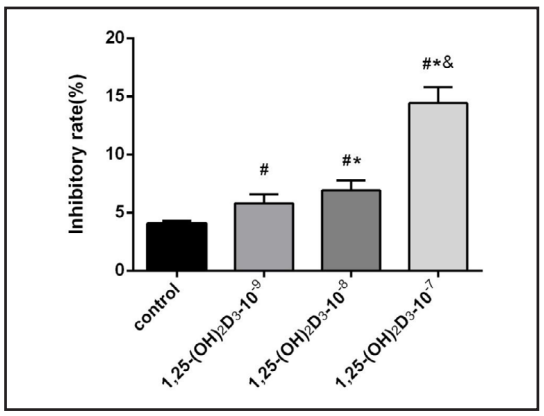

Fig. 3. Comparison of the proliferation inhibitory rate of fibroblast-like synoviocytes with different concentrations of 1,25-$(OH)_2D_3$: *, $P< 0.05$ in comparison tothe FLS treated with 1,25-$(OH)_2D_3$-10^{-9}; #, $P< 0.05$ in comparison totheFLS without treatment of 1,25-$(OH)_2D_3$; &, $P< 0.05$ in comparison totheFLS treated with 1,25-$(OH)_2D_3$-10^{-8}.

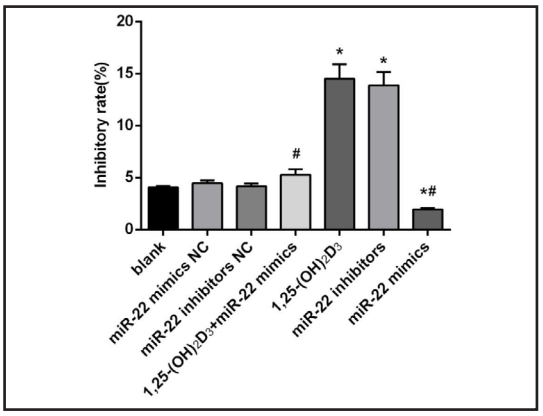

Fig. 4. Comparison of proliferationinhibitory rate of fibroblast-like synoviocytes among the seven groups: *, $P< 0.05$ in comparison tothe blank group, #, $P< 0.05$ in comparison to the 11,25-$(OH)_2D_3$ group.

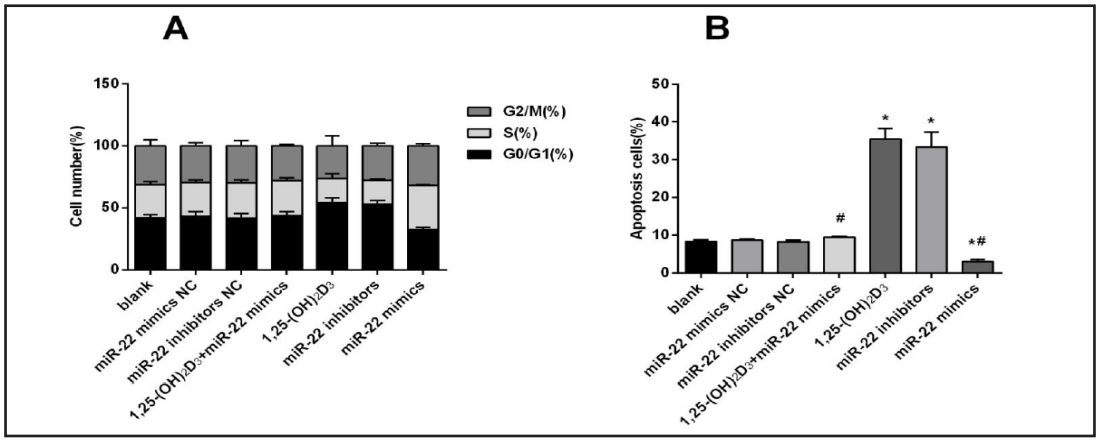

Fig. 5. Comparisons of cell cycle and apoptosis of fibroblast-like synoviocytes among the seven groups: A, cell cycle distribution of fibroblast-like synoviocytesin each group; B, apoptosis of fibroblast-like synoviocytesin each group; *, $P< 0.05$ in comparison to the blank group, #, $P< 0.05$ in comparison to the 1,25-$(OH)_2D_3$ group.

The optimal concentration of 1,25-$(OH)_2D_3$ estimated by MTT

According to the results of MTT, 1,25-$(OH)_2D_3$ had a inhibitory effect on the proliferation of FLS in the dose-dependent manner ($P< 0.05$), and the effect increased with an increased dose of 1,25-$(OH)_2D_3$. Therefore, the optimal concentration of 1,25-$(OH)_2D_3$ (10^{-7}mol/L) that showed the greatest inhibitory effect was selected for further experiments (Fig. 3).

Comparison of FLS proliferation among the seven groups

There was no significant difference in the proliferation inhibitory rate among the blank, miR-22 mimics NC, miR-22 inhibitors NC, and 1,25-$(OH)_2D_3$ + miR-22 mimics groups (all $P > 0.05$). Compared with the blank group, the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups showed an increased proliferation inhibitory rate, whereas the miR-22 mimics group showed a decreased rate (all $P<0.05$). Compared with the 1,25-$(OH)_2D_3$ group, the 1,25-$(OH)_2D_3$ + miR-22 mimics group had a decreased proliferation inhibitory rate ($P < 0.05$). These results suggested that 1,25-$(OH)_2D_3$ could inhibit FLS proliferation, which might be reversed by miR-22 mimics (Fig. 4).

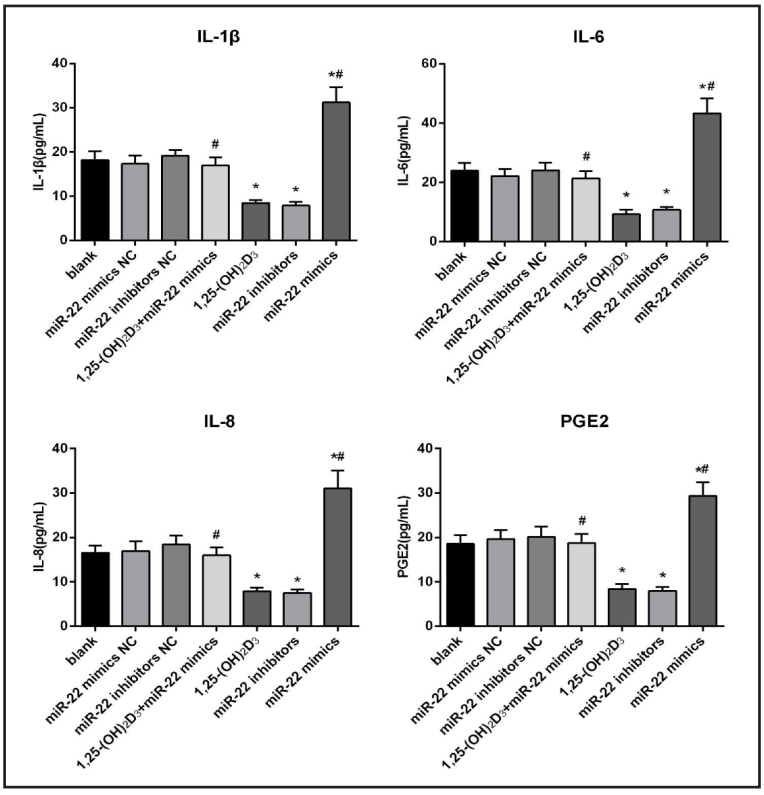

Fig. 6. Comparisons of pro-inflammatory cytokines protein expressions in fibroblast-like synoviocytes among the seven groups: *, $P< 0.05$ in comparison tothe blank group, #, $P< 0.05$ in comparison tothe 1,25-$(OH)_2D_3$ group.

Comparison of cell cycle and apoptosis among the seven groups

The results of cell cycle and apoptosis revealed that there was no notable difference among the blank, miR-22 mimics NC, miR-22 inhibitors NC and 1,25-$(OH)_2D_3$ + miR-22 mimics groups (all $P > 0.05$). Compared to the blank group, the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups had more cells at G0/G1 phase anda higher apoptosis rate, andthe miR-22 mimics group exhibited less cells at G0/G1 phase and a lower apoptosis rate ($P<0.05$). Compared to the 1,25-$(OH)_2D_3$ group, less cells at G0 / G1 phase and decreased apoptosis rate were observed in the 1,25-$(OH)_2D_3$ + miR-22 mimics group ($P< 0.05$) (Fig. 5).

Comparisons of the protein expressions of IL-1ß, IL-6, IL-8 and PGE2 among the seven groups

Based on the results of ELISA, no significant difference was observed in the protein expressions of pro-inflammatory cytokines (IL-I ß, IL-6, IL-8, and PGE2) among the blank, miR-22 mimics NC, miR-22 inhibitors NC, and 1,25-$(OH)_2D_3$ + miR-22 mimics groups (all $P > 0.05$). Compared with the blank group, the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups had down-regulated protein expressions of L-1ß, IL-6, IL-8 and PGE2, andthe miR-22 mimics group had higherprotein expressions of these cytokines (all $P< 0.05$). Compared with the 1,25-$(OH)_2D_3$ group, protein expressions of L-1ß, IL-6, IL-8, and PGE2 were up-regulated in the 1,25-$(OH)_2D_3$ + miR-22 mimics group ($P < 0.05$). It can be concluded that 1,25-$(OH)_2D_3$ could down-regulate the expressions of pro-inflammatory cytokines in FLS of rats with RA, which can be reversed by miR-22 mimics (Fig. 6).

Comparisons of mRNA expressions of miR-22, MMP-3, INOS and cox-2 among the seven groups

According to the qRT-PCR, there was no significant difference in the mRNA expressions of miR-22, MMP-3, INOS, and Cox-2 among the blank, miR-22 mimics NC, miR-22 inhibitors NC, and 1,25-$(OH)_2D_3$ + miR-22 mimics groups (all $P > 0.05$). Compared with the blank group, the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups had decreased mRNAexpressions of miR-22, MMP-3, INOS, and Cox-2, while mRNA expressions of miR-22, MMP-3, INOS, and Cox-2 were

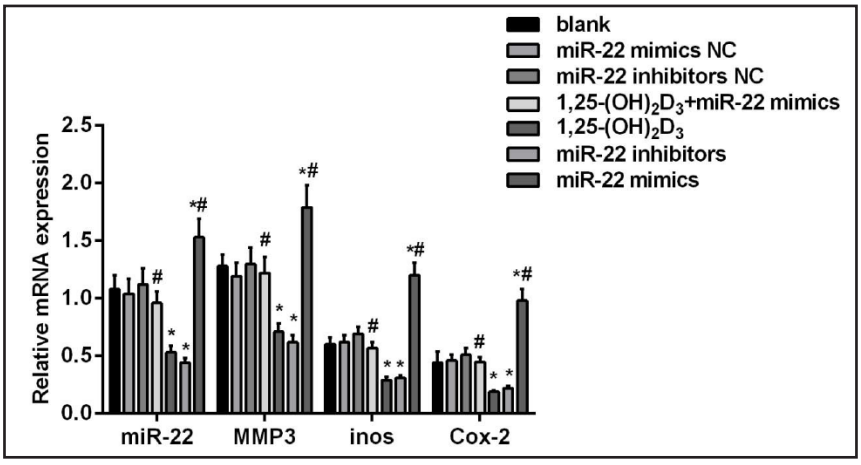

Fig. 7. Comparisons of mRNA expressions of miR-22, MMP-3, INOS and Cox-2 in fibroblast-like synoviocytes among the seven groups: *, $P< 0.05$ in comparison to the blank group, #, $P< 0.05$ in comparison to the 1,25-$(OH)_2D_3$ group.

elevated in the miR-22 mimics group (all $P<0.05$). Compared with the 1,25-$(OH)_2D_3$ group, the 1,25-$(OH)_2D_3$ + miR-22 mimics group showed increased mRNA expressions of miR-22, MMP-3, INOS, and Cox-2 ($P< 0.05$) (Fig. 7).

Discussion

RA, as a chronic inflammatory and autoimmune disease, is characterized by joint swelling, tenderness, and damage to synovial joints, resulting in severe disability and premature death [18]. Interestingly, it has been recognized that clinical outcomes of RA can be improved by early therapeutic intervention, which may also reduce the progression of joint damage and physical disability [19]. Current therapies, such as corticosteroids, are beneficial for inflammatory arthritis, but trigger systemic bone loss. Recently, Vitamin D supplements have been commonly provided to RA patients who take corticosteroids for the prevention of corticosteroid-induced osteoporosis, but its underlying mechanism is still unknown [20]. Researchers hold the idea that a greater intake of vitamin D is related to a lower risk of RA This is demonstrated in murine models of RA, where rats treated with active vitamin D displayed a decrease in the incidence rate and severity of RA [21].The purpose of the present study is to investigate the potential role of 1,25-$(OH)_2D_3$ on the proliferation of FLS in RA.

In this study, a significant increase of the proliferation inhibitory rate of FLS was observed in the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups when compared with the blank group, and no difference was seen in the FLS proliferation inhibitory rate between the 1,25-$(OH)_2D_3$ + miR-22 mimics and blank groups, indicating that 1,25-$(OH)_2D_3$ exerts an inhibitory function on the proliferation of FLS by down-regulating miR-22. RA FLS can exhibit resistance to apoptosis caused by apoptotic stimuli, further facilitating FLS hyperplastic growth and the destruction of articular cartilage [22]. Therefore, inhibiting the proliferation of RA FLS and prpmoting apoptosis of FLS are therapeutic approaches for the treatment of RA. 1,25-$(OH)_2D_3$(also known as calcitriol) functions by binding to and activating the nuclear vitamin D receptor (VDR), and nearly 3–5% of human genes are regulated by 1,25-$(OH)_2D_3$ directly or indirectly[23], through rapid signal transduction (the membrane receptor 1,25D_3-MARRS) [24]. 1,25D3-MARRS, a 57 kDa protein, has been recently identified as a membrane receptor for 1,25-$(OH)_2D_3$, through which 1,25-$(OH)_2D_3$ promotes the differentiation of NB4 promyelocytic leukemia cells [25]. More importantly, 1,25D3-MARRS, active in rat epithelia and chicken and mammalian bone development, shows positive-cooperativity in binding of 1,25-$(OH)_2D_3$ [26]. Coleman et al. have reported that the physiological function of membrane-initiated action of 1,25-$(OH)_2D_3$is limited, but mechanisms might be explained with 1,25-$(OH)_2D_3$-mediated signal transduction in growth inhibition. This is likely followed by VDR mediated transcriptional regulation of proliferation [27].

The results also showed that FLS at the G0/G1 stage were increased, and a highly elevated apoptosis rate of the FLS was observed in the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups. This suggests that 1,25-$(OH)_2D_3$ governs its own metabolism through regulating 24-hydroxylase activity, which results in the degradation of the molecule, and intermediate products (24-hydroxylated forms of 25-$(OH)D_3$ and 1,25-$(OH)2D_3$) might be biologically active during this reaction[28]. Bartels *et al.*showed that not only do the immune regulatory effects of the 1,25-$(OH)_2D_3$ area result in its action on antigen-presenting cells, but it directly inhibits the effects of 1,25-$(OH)_2D_3$ on human T cell proliferation and cytokine production induced by T cells [29]. Hager *et al.*'s study demonstrated that the biologically active form of 1,25-$(OH)_2D_3$can directly regulate the expression of p21 and p27, inducing a G0/G1 phase arrest [30]. To confirm this result, Alvarez-Diaz et al. showed that exposure to 1,25-$(OH)_2D_3$ results in the accumulation of cells in the G0/G1 phase of the cell cycle [14].

Additionally, expressions of pro-inflammatory cytokines including IL-Iβ, IL-6, IL-8, and PGE2 were relatively reduced in the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups. As IL-1β contributes to the degeneration of articular cartilage [31] and Xu *et al*'s study showed that IL-29 could promote acute inflammatory response by promoting the secretion of inflammatory cytokines including IL-6 and IL-8 [32], it suggests that the treatment of 1,25-$(OH)_2D_3$ can alleviate the inflammatory response in RA. Villaggio *et al.* have suggested that 1,25-$(OH)_2D_3$ can down-regulate the pro-inflammatory cytokine production in human activated macrophages through significantly reducing aromatase activity. This is especially presented in an environment with estrogenic milieu such as in RA synovial tissue [33]. In a previous study by Colin *et al.*, 1,25-$(OH)_2D_3$ was shown to suppress the expressions of TNF, IL-17, and interferon-(IFN) and to promote the production of IL-4, IL-5, and IL-10 by peripheral blood mononuclear cells (PBMCs) or CD4 T cells from healthy subjects [20]. In addition, 1,25-$(OH)_2D_3$ enhances the development of IL-10-producing CD4 T cells from patients with multiple sclerosis and Crohn's disease, and reduces the number of IL-6- and IL-17-secreting cells [34].

Moreover, there were decreased mRNA expressions of miR-22, MMP-3, INOS, and Cox-2 in the 1,25-$(OH)_2D_3$ and miR-22 inhibitors groups, which indicated that 1,25-$(OH)_2D_3$ can down-regulate these expressions. In terms of the correlation between 1,25-$(OH)_2D_3$ and mRNA-22, miR-22 was identified as a target of 1,25-$(OH)_2D_3$ in human colon cancer cells, which mediate in part its inhibitory effect on cell proliferation and migration [14]. Although some previous studies supported this association between miR-22 and 1,25-$(OH)_2D_3$, some showed the opposite result. The 1,25-$(OH)_2D_3$ can up-regulate the expression of miR-22 in ovarian cancer cells [35], and miR-22 inhibition promotes cell migration and reduces the anti-migratory effect of 1,25-$(OH)_2D_3$ in colon cancer [14]. Therefore, it is necessary to further explore the association between miR-22 and 1,25-$(OH)_2D_3$to confirm this result. As we found no difference in the mRNA expressions of miR-22, MMP-3, INOS, and Cox-2 between the 1,25-$(OH)_2D_3$ + miR-22 mimics and blank groups, it can be concluded that over-expression of miR-22 can reverse the effects of 1,25-$(OH)_2D_3$ on FLS. However, further studies are needed to confirm this.

Overall, our data demonstrates that 1,25-$(OH)_2D_3$ down-regulates miR-22 to inhibit the proliferation of FLS and promotes the apoptosis of FLS. This will hopefully encourage future studies to uncover the potential pathogenic mechanism of RA and provide a solid theoretical basis for developing effective and novel therapies for the clinical treatment of RA. However, further investigation is still needed to confirm the targeting correlation between 1,25-$(OH)_2D_3$ and miR-22.

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Disclosure Statement

None.

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